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Detection of *Borrelia bissettii* in Cardiac Valve Tissue of a Patient with Endocarditis and Aortic Valve Stenosis in the Czech Republic[▽]

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Molecular analysis of a clinical sample confirmed the presence of *Borrelia bissettii* DNA in cardiac valve tissue from a patient with endocarditis and aortic valve stenosis. This evidence strongly supports the involvement of *B. bissettii* in Lyme disease in Europe.

CASE REPORT

A 59-year-old white male patient was admitted to the hospital of České Budějovice in the Czech Republic in February 2007 for combined aortic valve disease. He was a construction engineer, social drinker, and ex-smoker with a positive family history of coronary artery disease in first-degree relatives (his father died of emboli at the age of 71). The patient presented with dyspnea and pain behind the sternum, which had been occurring for several months, mainly during fast walking upstairs. The patient reported having no symptoms when he was motionless. He had had a history of hyperbilirubinemia since 1999, and he also had a history of hypercholesteremia during the same period. Six months before he was admitted to the hospital, the patient was diagnosed with Lyme borreliosis and tick-borne meningoencephalitis on the basis of serological test results and clinical symptoms. He was cured after a course of antibiotics. Serological tests of blood drawn at the time of his hospital admission were positive for immunoglobulin G (IgG) antibodies and negative for IgM antibodies (data not shown) against *B. burgdorferi* and were confirmed by commercially available Western blot tests (ID blot borrelia IgG/ID blot borrelia IgM; Diagnostic Products Corporation) and interpreted by using CDC criteria (http://www.cdc.gov/mmwr/preview/mmwrhtml/00038469.htm). On examination, there were no other symptom of endocarditis, and the patient was hemodynamically stable.

Catheterization was conducted under nonemergency conditions in a single-procedure suite in the ward and revealed coronary artery disease and aortic regurgitation. The results of echocardiography revealed severe aortic stenosis, with a calculated orifice area of 0.80 cm^2 (i.e., 0.39 cm^2/m^2), second-degree aortic regurgitation, left ventricular hypertrophy, and left ventricular dilation (50 mm). The tricuspid aortic valve was heavily calcified, and mobility of the cusps was limited. A bicycle ergonometric test was performed but was stopped after 1 min due to tiredness, breathlessness, and muscle pain. Pain in the chest did not occur. An electrocardiogram showed permanent blockage of the left Tawarov shoulder. Hematological investigations revealed a hemoglobin level of 11.4 g/dl, a platelet count of 241×10^9/liter, and a white cell count of 5.5×10^9/liter. The initial interpretation of these data suggested a combined aortic stenosis and left bundle branch blockage. The patient was recommended for replacement of the aortic valve with a bioprosthesis (pericardial tissue heart valve, model 3000, size 27 mm; Edwards Lifesciences). Surgery confirmed the presence of atherosclerotic changes in the coronary artery and a highly calcified resected cardiac valve. Calcification presented in the cardiac conduction system as well. The surgery was completed with good hemodynamic parameters.

In the postoperative period, the atrioventricular (AV) heart block progressed from first to third degree, and it was necessary to implant a permanent cardiostimulator (6th day). The removed valve material was sent for microscopy and culture. Microscopic examination showed valve destruction and calcifications and the presence of solitary *Corynebacterium* cells. The cardiac valve tissue proved to be negative for aerobic and anaerobic microorganisms when cultured on blood agar and in Vf broth (Imuna Pharm a.s., Slovak Republic), respectively. Due to the Lyme disease in the anamnesis of the patient, a fragment of tissue sliced from the replaced valve was incubated in BSKH complete medium (Sigma) for cultivation of the Lyme disease spirochete. After 8 weeks of cultivation, the presence of live spirochetes in the medium was not confirmed either by dark-field microscopy or by PCR.

The remaining sample of replaced valve tissue was sent for molecular analysis. Processing of the sample involved direct DNA purification from the valve tissue (QIAamp tissue kit; Qiagen), PCR amplification of the *flagellin* gene with primers designed for *Borrelia burgdorferi* sensu lato (4) (FlaF, 5′-AARGAATTGGCAGTTCAATC-3′, and FlaR, 5′-GCATTTTCWATTTTAGCAAGTGATG-3′, where "R" indicates an A to G substitution and "W" indicates an A to T substitution), analysis of the PCR product and its purification (QIAquick gel extraction kit; Qiagen), cloning into a pCR4-TOPO vector (Topo TA cloning kit for sequencing; Invitrogen), amplicon sequencing

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TABLE 1. RFLP analysis of partial *flagellin* gene from Lyme disease complex spirochetes and isolate of human origin from the Czech Republic

Organism	Size of *flagellin* sequence (bp)	In silico RFLP patterns for indicated restriction enzyme				
		CelII	DdeI	HapII	HhaI	HincII
Control *Borrelia* spp.						
B. burgdorferi B31	488		338, 150	349, 139		
B. afzelii VS461	488	380, 108	305, 108, 42, 33			
B. andersonii 21133	488		238, 150, 100			
B. bissettii DN127	487		220, 117, 78, 72		404, 83	
B. californiensis 443[a]	456		225, 117, 111, 3[a]		281, 175[a]	
B. garinii 20047	488		329, 78, 72, 9		405, 83	453, 35
B. japonica HO14	488		305, 150, 33		348, 140	
B. lusitaniae PotiB2	488		338, 150		393, 83, 12	453, 35
B. sinica CMN3	488		338, 150		300, 105, 83	453, 35
B. spielmanii (A14S)	488		338, 150		348, 140	
B. tanukii Hk501	488		338, 150		256, 232	
B. turdi Ya501	488		221, 150, 117		405, 83	453, 35
B. valaisiana VS116	488		188, 135, 117, 33, 15			
flagellin isolate from patient						
p18E12 (*B. bissettii*)	487		220, 117, 78 (45 + 27)		404, 83	

[a] The size of the partial *flagellin* sequence of *B. californiensis* (GenBank accession number DQ393346) differs significantly from those of the rest of the species.

from both sides with the M13F/M13R universal primers, sequence analysis with DNAStar software, database searches using the BLAST programs of the NCBI (Bethesda, MD), in silico restriction fragment length polymorphism (RFLP) analysis for *flagellin*, and phylogenetic analysis. To avoid any contaminants, the reactions were set up in a separate area, and all relevant precautions regarding supplies, equipment, safety items for the personnel, and pre- and postamplification activities were taken.

All the negative controls (no template) were negative. DNA from *B. burgdorferi* B31 was used as a positive control. PCR amplification resulted in a 487-bp-long *flagellin* amplicon. A preliminary search for similar sequences in GenBank showed 99% identity to the *flagellin* gene of *Borrelia bissettii* strain DN127 (GenBank accession number D82858). The PCR product obtained is not likely due to contamination, as we had not conducted analyses with any strain related to *B. bissettii* prior to this study. The detected nucleotide substitutions (3 out of 487 bp) could be explained by the well-known diversity within *B. bissettii* strains. The RFLP analysis of the *Borrelia flagellin* sequences was done in silico, using free software available at http://insilico.ehu.es (2). *flagellin* sequences of all 13 species of the *B. burgdorferi* sensu lato complex were used as controls and were obtained from GenBank. The sequences of the *flagellin* gene amplified from the total DNA of the cardiac valve tissue were digested by using the restriction sites for HapII, HhaI, HincII, CelII, and DdeI, and the obtained in silico RFLP pattern was compared with the already published *flagellin* patterns of 97 different strains representing a total of 22 spirochete species from two groups, Lyme disease spirochetes and relapsing-fever borrelia (5). The in silico RFLP pattern of the *flagellin* gene amplified from the cardiac valve tissue is identical to that of *B. bissettii* DN127 (Table 1).

The phylogenetic analysis (Fig. 1) was performed with PAUP* by implementing the tree bisection/reconnection algorithm. The alignments were done with ClustalX (version 1.81) (18). Identical sequences were excluded from the analysis. Gaps were treated as missing characters. Branch supports were calculated by bootstrap analyses (1,000 replicates for a molecular data set). Results were confirmed by using the maximum likelihood method. The *flagellin* sequence amplified from the human cardiac valve tissue clustered together with the control *B. bissettii* DN127 sequence and was separate from that of any other species of Lyme disease spirochetes. In conclusion, a sequence similarity search of the partial *flagellin* gene amplified from the total DNA of the valve tissue, *flagellin* RFLP pattern analysis, and phylogenetic analysis confirmed the presence of *B. bissettii* DNA in the cardiac valve tissue of the patient with endocarditis and aortic valve stenosis.

Lyme borreliosis, the most common vector-borne disease in Europe and the United States, is a multisystemic infection caused by *B. burgdorferi* sensu lato spirochetes (14). Of the thousands of Lyme disease cases each year, up to 10% result in cardiac complications (1). Lyme disease is well known for affecting the myocardium in the form of carditis and dilated cardiomyopathy. Infectious endocarditis is a rare life-threatening disease. Multiple infectious agents have been associated with carditis. The most commonly identified agents are viruses, most notably the coxsackie B virus, but several bacteria, protozoa, and fungi can also cause carditis (7, 9, 10).

Spirochetes are one group of bacteria with a predilection for cardiac infection. The use of a broad-range bacterial PCR followed by direct sequencing has been successful in the detection of spirochete DNA in excised cardiac tissues of patients with infective endocarditis (3, 6) and culture-negative infective endocarditis (17). Borrelial endocarditis can represent a specific problem due to its various forms, which are difficult to diagnose (especially if the disease manifests itself in ways other than atrioventricular AV blockade) due to difficulties in detecting fastidious pathogens or pathogens from patients that have been pretreated with antibiotics, which cannot be cultivated, thus preventing identification by culture.

Various degrees of AV conduction block are the most com-

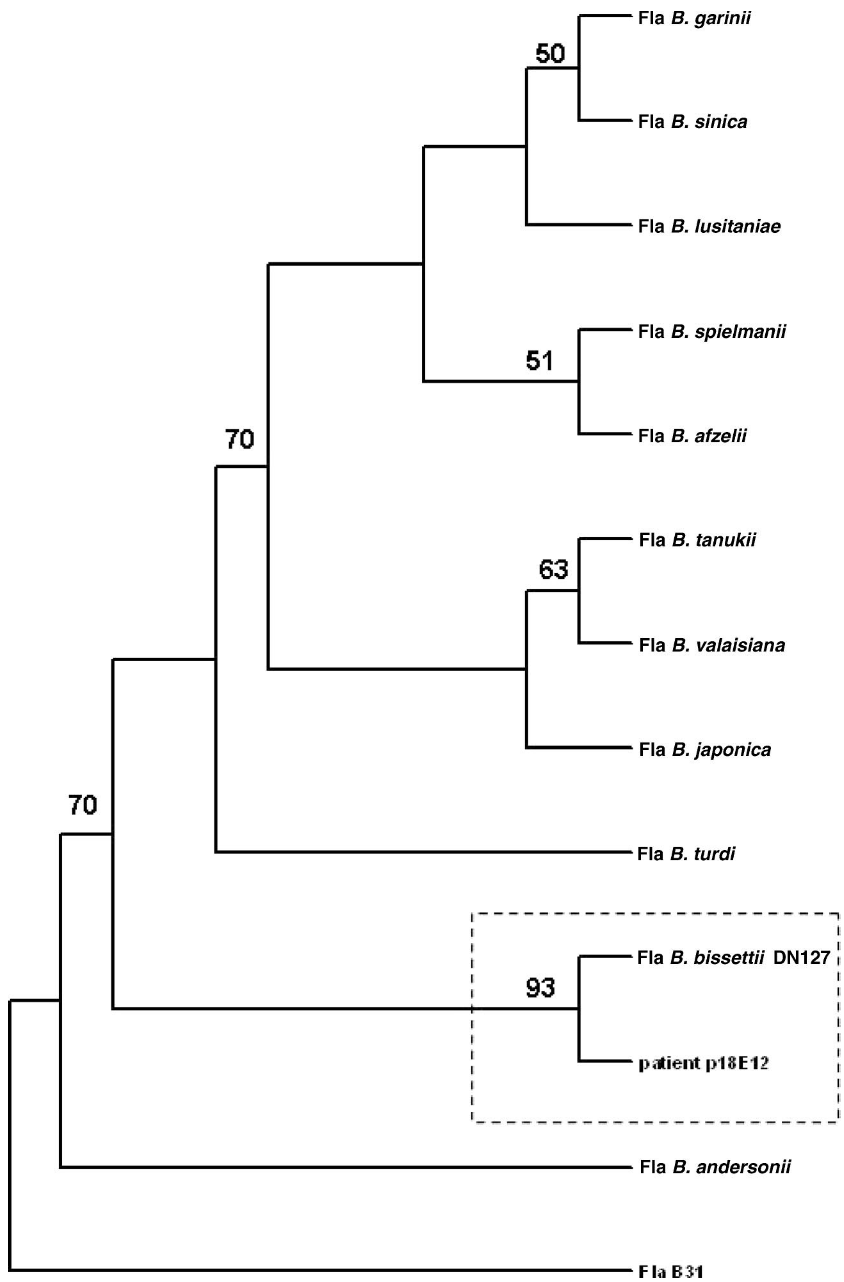

FIG. 1. Phylogenetic analysis of partial *flagellin* sequences from Lyme disease complex spirochetes and from the cardiac valve tissue of a patient from the Czech Republic with endocarditis. Sequences of the *flagellin* gene from 12 control species of the *B. burgdorferi* sensu lato complex were downloaded from GenBank (accession numbers: *B. afzelii*, D63365; *B. andersonii*, D83764; *B. bissettii*, D82857; *B. burgdorferi* B31, X16933; *B. garinii*, D82846; *B. japonica*, D82852; *B. lusitaniae*, D82856; B. *spielmanii*, DDQ111034; *B. sinica*, AB022138; *B. tanukii*, D82847; *B. turdi*, D82849; and *B. valaisiana*, D82854). *B. californiensis* (GenBank accession number DQ393346) was excluded from the phylogenetic analysis due to the inappropriate size of the *flagellin* sequence available in GenBank.

mon manifestations of Lyme carditis. Patients presenting with first-degree AV blocks can rapidly progress to second-degree or complete heart blocks (11). We presented here a case of third-degree AV block in a patient with Lyme disease in anamnesis, with negative results from bacterial cultivation but confirmation by molecular techniques of the presence of *B. bissettii* DNA in the valve tissue. This case raises the question of whether DNA persists without any evidence of infection (13). The disadvantage of DNA-based methods in pathogen detection is that they do not distinguish between living and dead organisms (8). Although we cannot state that the DNA detected in our case does predict the occurrence of infectious endocarditis, the results show that DNA from the causative agent of Lyme disease may persist in the cardiac valves of patients some time after the infection is cured by a course of antibiotics. Our case also provides evidence that *B. bissettii*, first isolated from the lymphocytoma tissue of a patient in Slovenia (12, 15, 16) and then detected in cardiac valve tissue from the Czech patient, is involved in Lyme disease of humans in Europe. The presence of *B. bissettii* as the single *Borrelia* strain in patients with symptomatic borreliosis or chronic borrelial infections strongly supports the fact that *B. bissettii* may indeed be a causative agent of Lyme disease.

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